A NEW CLASS OF ENZYMES IN THE BIOSYNTHETIC PATHWAY FOR THE PRODUCTION OF TRIACYLGLYCEROL AND RECOMBINANT DNA MOLECULES ENCODING THESE ENZYMES

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The present invention relates to the isolation, identification and characterization of recombinant DNA molecules encoding enzymes catalysing the transfer of fatty acids from phospholipids to diacylglycerol in the biosynthetic pathway for the production of triacylglycerol.

Triacylglycerol (TAG) is the most common lipid-based energy reserve in nature. The main pathway for synthesis of TAG is believed to involve three sequential acyl-transfers from acyl-CoA to a glycerol backbone (1, 2). For many years, acyl-CoA : diacylglycerol acyltransferase (DAGAT), which catalyzes the third acyl transfer reaction, was thought to be the only unique enzyme involved in TAG synthesis. It acts by diverting diacylglycerol (DAG) from membrane lipid synthesis into TAG (2). Genes encoding this enzyme were recently identified both in the mouse (3) and in plants (4, 5), and the encoded proteins were shown to be homologous to acyl-CoA : cholesterol acyltransferase (ACAT). It was also recently reported that another DAGAT exists in the oleaginous fungus *Mortierella ramanniana,* which is unrelated to the mouse DAGAT, the ACAT gene family or to any other known gene (6).

The instant invention relates to novel type of enzymes and their encoding genes for transformation. More specifically, the invention relates to use of a type of genes encoding a not previously described type of enzymes hereinafter designated phospholipid:diacylglycerol acyltransferases (PDAT), whereby this enzyme catalyses an acyl-CoA-independent reaction. The said type of genes expressed alone in transgenic organisms will enhance the total amount of oil (triacylglycerols) produced in the cells. The PDAT genes, in combination with a gene for the synthesis of an uncommon fatty acid will, when expressed in transgenic organisms, enhance the levels of the uncommon fatty acids in the triacylglycerols.

There is considerable interest world-wide in producing chemical feedstock, such as fatty acids, for industrial use from renewable plant resources rather than non-renewable petrochemicals. This concept has broad appeal to manufacturers and consumers on the basis of resource conservation and provides significant opportunity to develop new industrial crops for agriculture.

There is a diverse array of unusual fatty acids in oils from wild plant species and these have been well characterised. Many of these acids have industrial potential and this has led to interest in domesticating relevant plant species to enable agricultural production of particular fatty acids.

Development in genetic engineering technologies combined with greater understanding of the biosynthesis of unusual fatty acids now makes it possible to transfer genes coding for key enzymes involved in the synthesis of a particular fatty acid from a wild species into domesticated oilseed crops. In this way individual fatty acids can be produced in high purity and quantities at moderate costs.

In all crops like rape, sunflower, oilpalm etc., the oil (i.e. triacylglycerols) is the most valuable product of the seeds or fruits and other compounds like starch, protein, and fibre is regarded as by-products with less value. Enhancing the quantity of oil per weight basis at the expense of other compounds in oil crops would therefore increase the value of crop. If genes, regulating the allocation of reduced carbon into the production of oil can be up-regulated, the cells will accumulate more oil on the expense af other products. Such genes might not only be used in already high oil producing cells such as oil crops but could also induce significant oil production in moderate or low oil containing crops such as e.g. soy, oat, maize, potato, sugarbeats, and turnips as well as in micro-organisms.

Summary of the invention

Many of the unusual fatty acids of interest, e.g. medium chain fatty acids, hydroxy fatty acids, epoxy fatty acids and acetylenic fatty acids, have physical properties that are distinctly different from the common plant fatty acids. The present inventors have found that, in plant species naturally accumulating these uncomman fatty acids in their seed oil (i.e. triacylglycerol), these acids are absent, or present in very low amounts in the membrane (phospho)lipids of the seed. The low concentration of these acids in the membrane lipids is most likely a prerequisite for proper membrane function and thereby for proper cell functions. One aspect of the invention is that seeds of transgenic crops can be made to accumulate high amounts of uncommon fatty acids if these fatty acids are efficiently removed from the membrane lipids and channelled into seed triacylglycerols.

The inventors have identified a novel class of enzymes in plants catalysing the transfer of fatty acids from phospholipids to diacylglycerol in the production of triacylglycerol through an acyl-CoA-independent reaction and that these enzymes (phospholipid:diacylglycerol acyltransferases abbreviated as PDAT) are involved in the removal of hydroxylated, epoxygenated fatty acids, and probably also other uncommon fatty acids such as medium chain fatty acids, from phospholipids in plants.

This enzyme reaction was shown to be present in microsomal preparations from baker's yeast (*Saccharomyces cerevisiae*). The instant invention further pertains to an enzyme comprising an amino acid sequence as set forth in SEQ ID No. 2 or a functional fragment, derivate, allele, homologue or isoenzyme thereof. A so called ‚knock out' yeast mutant, disrupted in the respective gene was obtained and microsomal membranes from the mutant was shown to totally lack PDAT activity. Thus, it was proved that the disrupted gene encodes for a PDAT enzyme (SEQ ID NO. 1 and 2).

The instant invention pertains further to an enzyme comprising an amino acid sequence as set forth in SEQ ID NO. 1a, 2b or 5a or a functional fragment, derivate, allele, homologue or isoenzyme thereof.

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Further genes and/or ~~peoteins~~ proteins of so far unknown function were identified and are contemplated within the scope of the instant invention. A gene from Schizosaccharomyces pombe, SPBC776.14 (SEQ ID. NO. 3), a putative open reading frame CAA22887 of the SPBC776.14 (SEQ ID NO. 13) were identified. Further Arabidopsis thaliana genomic sequences (SEQ ID NO. 4, 10 and 11) coding for putative proteins were identified, as well as a putative open reading frame AAC80628 from the A. thaliana locus AC 004557 (SEQ ID NO. 14) and a putative open reading frame AAD10668 from the A. thaliana locus AC 003027 (SEQ ID NO. 15) were identified.

Also, a partially sequenced cDNA clone from Neurospora crassa (SEQ ID NO. 9) and a Zea mays EST (Extended Sequence Tac) clone (SEQ ID NO. 7) and corresponding putative amino acid sequence (SEQ ID NO. 8) were identified. Finally, two cDNA clones were identified, one Arabidopsis thaliana EST (SEQ ID NO. 5 and corresponding predicted amino acid sequence SEQ ID NO. 6) and a Lycopersicon esculentum EST clone (SEQ ID NO. 12) were identified. Further, enzymes designated as PDAT comprising an amino acid sequence selected from the group consisting of sequences as set forth in SEQ ID NO 2a, 3a, 5b, 6 or 7b are contemplated within the scope of the invention. Moreover, an enzyme comprising an amino acid sequence encoded through a nucleotide sequence, a portion, derivate, allele or homologue thereof selected from the group consisting of sequences as set forth in SEQ ID No. 1, 1b, 3, 3b, 4, 4a, 4b, 5, 5b, 6b, 7, 8b, 9, 9b, 10, 10b, 11, 11b or 12 or a functional fragment, derivate, allele, homologue or isoenzyme of the enzyme encoding amino acid sequence are included within the scope of the invention.

A fuctional fragment of the instant enzyme is understood to be any polypeptide sequence which shows specific enzyme activity of a phospholipid:diacylglycerol acyltransferase (PDAT). The length of the functional fragment can for example

vary in a range from about 660 ± 10 amino acids to 660 ± 250 amino acids, preferably from about 660 ± 50 to 660 ±100 amino acids, whereby the „basic number" of 660 amino acids corresponds in this case to the polypeptide chain of the PDAT enzyme of SEQ ID NO. 2 encoded by a nucleotide sequence according to SEQ ID NO. 1. Consequently, the „basic number" of functional ~~fullength~~ full length enzyme can vary in correspondance to the encoding nucleotide sequence.

A portion of the instant nucleotide sequence is meant to be any nucleotide sequence encoding a ~~polypeptid~~ polypeptide which shows specific activity of a phospholipid:diacylglycerol acyltransferase (PDAT). The length of the nucleotide portion can vary in a wide range of about several ~~hundereds~~ hundreds of nucleotides based upon the coding region of the gene or a highly conserved ~~sequnence~~ sequence. For example the length varies in a range form about 1900 ± 10 to 1900 ± 1000 nucleotides, preferably form about 1900 ± 50 to 1900 ±700 and more preferably ~~form~~ from about 1900 ± 100 to 1900 ± 500 nucleotides. whereby the „basic number" of 1900 ~~nucleotdies~~ nucleotides corresponds in this case to the encoding nucleotide sequence of the PDAT enzyme of SEQ ID NO. 1. Consequently, the „basic number" of functional ~~fullength~~ full length gene can vary.

An allelic variant of the instant nucleotide sequence is understood to be any different nucleotide sequence which encodes a polypeptide with a functionally equivalent function. The alleles pertain naturally occuring variantes of the instant nucleotide sequences as well as synthetic nucleotide sequences produces by methods known in the art. Contemplated are even altered nucleotide sequences which result in an enzyme with altered activity and/or regulation or which is resistant against specific inhibitors. The instant invention further includes natural or synthetic mutations of the originally isolated nucleotide sequences. These mutations can be substitution, addition, deletion, inversion or insertion of one or more nucleotides.

A homologues nucleotide sequence is understood to be a complementary sequence and/or a sequence which specifically hybridizes with the instant nucleotide sequence. Hybridizing sequences include similar sequences selected from the group of DNA or RNA which specifically interact to the instant nucleotide sequences under at least moderate stringency conditions which are known in the art. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. This further includes short nucleotide sequences of e.g. 10 to 30 nucleotides, preferably 12 to 15 nucleotides. Included are also primer or hybridization probes.

A homologue nucleotide sequence included within the scope of the instant invention is a sequence which is at least about 40%, preferably at least about 50 % or 60%, and more preferably at least about 70%, 80% or 90% and most preferably at least about 95%, 96%, 97%, 98% or 99% or more homologous to a nucleotide sequence of SEQ ID NO. 1.
All of the aforementioned definitions are true for amino acid seqences and functional enzymes and can easily transfered by a person skilled in the art.

Isoenzymes are understood to be enzymes which have the same or a similar substrate specifity and/or catalytic activity but a different primary structure.

In a first embodiment, this invention is directed to nucleic acid sequences that encode a PDAT. This includes sequences that encode biologically active PDATs as well as sequences that are to be used as probes, vectors for transformation or cloning intermediates. The PDAT encoding sequence may encode a complete or partial sequence depending upon the intended use. All or a portion of the genomic sequence, cDNA sequence, precursor PDAT or mature PDAT is intended.

Further included is a nucleotide sequence selected from the group consisting of sequences set forth in SEQ ID No. 1, 1b, 3, 3b, 4, 4a, 4b, 9b, 10, 10b or 11 or a portion, derivate, allele or homologue thereof. The invention pertains a partial nucleotide sequence corresponding to a fullength nucleotide sequence selected from the group consisting of sequences set forth in SEQ ID No. 5, 5b, 6b, 7, 8b, 9, 11b or 12 or a portion, derivate, allele or homologue thereof. Moreover, a nucleotide sequence comprising a nucleotide sequence which is at least 40% homologous to a nucleotide sequence selected form the group consisting of those sequences set forth in SEQ ID No. 1 1b, 3, 3b, 4, 4a, 4b, 5, 5b, 6b, 7, 8b, 9, 9b, 10, 10b, 11, 11b or 12 is contemplated within the scope of the invention.

The instant invention pertains to a gene construct comprising a said nucleotide sequences of the instant invention which is operably linked to a heterologous nucleic acid.
The term operably linked means a serial organisation e.g. of a promotor, coding sequence, terminator and/or further regulatory elements whereby each element can fulfill its original function during expression of the nucleotide sequence.

Further, a vector comprising of a said nucleotide sequence of the instant invention is contemplated in the instant invention. This includes also an expression vector as well as a vector further comprising a selectable marker gene and/or nucleotide sequences for the replication in a host cell and/or the integration into the genome of the host cell.

In a different aspect, this invention relates to a method for producing a PDAT in a host cell or progeny thereof, including genetically engineered oil seeds, yeast and moulds or any other oil accumulating organism, via the expression of a construct in the cell. Cells containing a PDAT as a result of the production of the PDAT encoding sequence are also contemplated within the scope of the invention.

Further, the invention pertains a transgenic cell or organism containing a said nucleotide sequence and/or a said gene construct and/or a said vector. The object of the instant invention is further a transgenic cell or organism which is an eucaryotic cell or organism. Preferably, the transgenic cell or organism is a yeast cell or a plant cell or a plant. The instant invention further pertains said transgenic cell or organism having an altered biosynthetic pathway for the production of triacylglycerol. A transgenic cell or organism having an altered oil content is also contemplated within the scope of this invention.

Further, the invention pertains a transgenic cell or organism wherein the activity of PDAT is altered in said cell or organism. This altered activity of PDAT is characterized by an alteration in gene expression, catalytic activity and/or regulation of activity of the enzyme. Moreover, a transgenic cell or organism is included in the instant invention, wherein the altered biosynthetic pathway for the production of triacylglycerol is characterized by the prevention of accumulation of undesirable fatty acids in the membrane lipids.

In a different embodiment, this invention also relates to methods of using a DNA sequence encoding a PDAT for increasing the oil-content within a cell.

Another aspect of the invention relates to the accommodation of high amounts of uncomman fatty acids in the triacylglycerol produced within a cell, by introducing a DNA sequence producing a PDAT that specifically removes these fatty acids from the membrane lipids of the cell and channel them into triacylglycerol. Plant cells having such a modification are also contemplated herein.

Further, the invention pertains a process for the production of triacylglycerol, comprising growing a said transgenic cell or organism under conditions whereby the said nucleotide sequence is expressed and whereby the said

transgenic cells comprising an said enzyme catalysing the transfer of fatty acids from phospholipids to diacylglycerol forming triacylglycerol.

Moreover, triacylglycerols produced by the aforementioned process are included in scope of the instant invention.

Object of the instant invention is further the use of an instant nucleotide sequence and/or a said enzyme for the production of triacylglycerol and/or triacylglycerols with uncommon fatty acids. The use of a said instant nucleotide sequence and/or a said enzyme of the instant invention for the transformation of any cell or organism in order to be expressed in this cell or organism and result in an altered, preferably increased oil content of this cell or organism is also contemplated within the scope of the instant invention.

A PDAT of this invention includes any sequence of amino acids, such as a protein, polypeptide or peptide fragment obtainable from a microorganism, animal or plant source that demonstrates the ability to catalyse the production of triacylglycerol from a phospholipid and diacylglycerol under enzyme reactive conditions. By "enzyme reactive conditions" is meant that any necessary conditions are available in an environment (e.g., such factors as temperature, pH, lack of inhibiting substances) which will permit the enzyme to function.

Other PDATs are obtainable from the specific sequences provided herein. Furthermore, it will be apparent that one can obtain natural and synthetic PDATs, including modified amino acid sequences and starting materials for synthetic-protein modelling from the exemplified PDATs and from PDATs which are obtained through the use of such exemplified sequences. Modified amino acid sequences include sequences that have been mutated, truncated, increased and the like, whether such sequences were partially or wholly synthesised. Sequences that are actually purified from plant preparations or are identical or encode identical proteins thereto, regardless of the method

used to obtain the protein or sequence, are equally considered naturally derived.

Further, the nucleic acid probes (DNA and RNA) of the present invention can be used to screen and recover "homologous" or "related" PDATs from a variety of plant and microbial sources.

Further, it is also apparent that a person skilled in the art can, with the information provided in this application, in any organism identify a PDAT activity, purify an enzyme with this activity and thereby identify a "non-homologues" nucleic acid sequence encoding such an enzyme.

The present invention can be essentially characterized by the following aspects:

1. Use of a PDAT gene (genomic clone or cDNA) for transformation.
2. Use of a DNA molecule according to item 1 wherein said DNA is used for transformation of any organism in order to be expressed in this organism and result in an active recombinant PDAT enzyme in order to increase oil content of the organism.
3. Use of a DNA molecule of item 1 wherein said DNA is used for transformation of any organism in order to prevent the accumulation of undesirable fatty acids in the membrane lipids.
4. Use according to item 1, wherein said PDAT gene is used for transforming transgenic oil accumulating organisms engineered to produce any uncommon fatty acid which is harmful if present in high amounts in membrane lipids, such as medium chain fatty acids, hydroxylated fatty acids, epoxygenated fatty acids and acetylenic fatty acids.
5. Use according to item 1, wherein said PDAT gene is used for transforming organisms, and wherein said organisms are crossed with other oil accummulating organisms engineered to produce any uncommon fatty acid

which is harmful if present in high amounts in membrane lipids, comprising medium chain fatty acids, hydroxylated fatty acids, epoxygenated fatty acids and acetylenic fatty acids.

6. Use according to item 1, wherein the enzyme encoded by said PDAT gene or cDNA is coding for a PDAT with distinct acyl specificity.
7. Use according to item 1 wherein said PDAT encoding gene or cDNA, is derived from *Saccharomyces cereviseae,* or contain nucleotide sequences coding for an amino acid sequence 30% or more identical to the amino acid sequence of PDAT as presented in SEQ. ID. NO. 2.
8. Use according to item 1 wherein said PDAT encoding gene or cDNA is derived from *Saccharomyces cereviseae,* or contain nucleotide sequences coding for an amino acid sequence 40% or more *identical* to the amino acid sequence of PDAT as presented in SEQ. ID. NO. 2.
9. Use according to item 1 wherein said PDAT encoding gene or cDNA is derived from *Saccharomyces cereviseae,* or contain nucleotide sequences coding for an amino acid sequence 60% or more *identical* to the amino acid sequence of PDAT as presented in SEQ. ID. NO. 2.
10. Use according to item 1 wherein said PDAT encoding gene or cDNA is derived from *Saccharomyces cereviseae,* or contain nucleotide sequences coding for an amino acid sequence 80% or more identical to the amino acid sequence of PDAT as presented in SEQ. ID. NO. 2.
11. Use according to item 1 wherein said PDAT encoding gene or cDNA is derived from plants or contain nucleotide sequences coding for an amino acid sequence 40% or more identical to the amino acid sequence of PDAT from *Arabidopsis thaliana* or to the protein encoded by the fullength counterpart of the partial Zea mays, Lycopericon esculentum, or Neurospora crassa cDNA clones.
12. Transgenic oil accumulating organisms comprising, in their genome, a PDAT gene transferred by recombinant DNA technology or somatic hybridization.

13. Transgenic oil accumulating organisms according to item 12 comprising, in their genome, a PDAT gene having specificity for substrates with a particular uncommon fatty acid and the gene for said uncommon fatty acid.
14. Transgenic organisms according to item 12 or 13 which are selected from the group consisting of fungi, plants and animals.
15. Transgenic organisms according to item 12 or 13 which are selected from the group of agricultural plants.
16. Transgenic organisms according to item 12 or 13 which are selected from the group of agricultural plants and where said PDAT gene is expressed under the control of a storage organ specific promotor.
17. Transgenic organisms according to item 12 or 13 which are selected from the group of agricultural plants and where said PDAT gene is expressed under the control of a seed promotor.
18. Oils from organisms according to item 12 – 17.
19. A method for altering acyl specificity of a PDAT by alteration of the nucleotide sequence of a naturally occurring encoding gene and as a consequence of this alternation creating a gene encoding for an enzyme with novel acyl specifity.
20. A protein encoded by a DNA molecule according to item 1 or a functional fragment thereof.
21. A protein of item 20 designated phospholipid:diacylglycerol acyltransferase.
22. A protein of item 21 which has a distinct acyl specificity.
23. A protein of item 13 having the amino acid sequence as set forth in SEQ, ID NO. 2, 13, 14 or 15 (and the proteins encoded by the fullength or partial genes set forth in SEQ. ID. NO. 1, 3, 4, 5, 7, 9, 10, 11 or 12) or an amino acid sequence with at least 30 % homology to said amino acid sequence.
24. A protein of item 23 isolated from Saccharomyces cereviseae.

<u>General methods:</u>

Yeast strains and plasmids. The wild type yeast strains used were either FY1679 (*MATα his3-Δ200 leu2-Δ1 trp1-Δ6 ura3-52*) or W303-1A (*MATa ADE2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) (7). The YNR008w::KanMX2 disruption strain FVKT004-04C(AL), which is congenic to FY1679, was obtained from the Euroscarf collection (8). A 2751 bp fragment containing the YNR008w gene with 583 bp of 5' and 183 bp of 3' flanking DNA was amplified from W303-1A genomic DNA using *Taq* polymerase with 5'-TCTCCATCTTCTGCAAAACCT-3' and 5'-CCTGTCAAAAACCTTCTCCTC-3' as primers. The resulting PCR product was purified by agarose gel electrophoresis and cloned into the *Eco*RV site of pBluescript (pbluescript-pdat). For complementation experiments, the cloned fragment was released from pBluescript by *Hin*dIII-*Sac*I digestion and then cloned between the *Hin*dIII and *Sac*I sites of pFL39 (9), thus generating pUS1. For overexpression of the PDAT gene, a 2202 bp *Eco*RI fragment from the pBluscript plasmid which contains only 24 bp of 5' flanking DNA was cloned into the BamHI site of the *GAL1-TPK2* expression vector pJN92 (12), thus generating pUS4.

Microsomal preparations. Microsomes from developing seeds of sunflower (*Helianthus annuus*), *Ricinus communis* and *Crepis palaestina* were prepared using the procedure of Stobart and Stymne (11). To obtain yeast microsomes, 1g of yeast cells (fresh weight) was re-suspended in 8 ml of ice-cold buffer (20 mM Tris-Cl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5 % (v/v) glycerol, 1 mM DTT, 0.3 M ammonium sulfate) in a 12 ml glass tube. To this tube, 4 ml of glass beads (diameter 0.45-0.5 mm) were added, and the tube was then heavily shaken (3 x 60 s) in an MSK cell homogenizer (B. Braun Melsungen AG, Germany). The homogenized suspension was centrifuged at 20,000 x g for 15 min at 6°C and the resulting supernatant was again centrifuged at 100,000 x g for 2 h at 6°C. The 100,000 x g pellet was resuspended in 0.1 M potassium phosphate (pH 7.2), and stored at -80°C. It is subsequently referred to as the crude yeast microsomal fraction.

Lipid substrates. Radio-labeled ricinoleic (12-hydroxy-octadecenoic) and vernolic (12,13-epoxy-octadecenoic) acids were synthesized enzymatically from [1-^{14}C]oleic acid and [1-^{14}C]linoleic acid, respectively, by incubation with microsomal preparations from seeds of *Ricinus communis* and *Crepis palaestina*, respectively (12). The synthesis of phosphatidylcholines (PC) or phosphatidylethanolamines (PE) with ^{14}C-labeled acyl groups in the *sn*-2 position was performed using either enzymatic (13), or synthetic (14) acylation of [^{14}C]oleic, [^{14}C]ricinoleic, or [^{14}C]vernolic acid. Dioleoyl-PC that was labeled in the *sn*-1 position was synthesized from *sn*-1-[^{14}C]oleoyl-lyso-PC and unlabeled oleic acid as described in (14). *Sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-DAG was synthesized from PC by the action of phospholipase C type XI from *B. Cereus* (Sigma Chemical Co.) as described in (15). Monovernoloyl- and divemoleoyl-DAG were synthesized from TAG extracted from seeds of *Euphorbia lagascae*, using the TAG-lipase (Rizhopus arrhizus, Sigma Chemical Co.) as previously described (16). Monoricinoleoyl-TAG was synthesized according to the same method using TAG extracted from Castor bean.

Lipid analysis. Total lipid composition of yeast were determined from cells harvested from a 40 ml liquid culture, broken in a glass-bead shaker and extracted into chloroform as described by Bligh and Dyer (17), and then separated by thin layer chromatography in hexane/diethylether/acetic acid (80:20:1) using pre-coated silica gel 60 plates (Merck). The lipid areas were located by brief exposure to I_2 vapors and identified by means of appropriate standards. Polar lipids, sterol-esters and triacylglycerols, as well as the remaining minor lipid classes, referred to as other lipids, were excised from the plates. Fatty acid methylesters were prepared by heating the dry excised material at 85 °C for 60 min in 2% (v/v) sulfuric acid in dry methanol. The methyl esters were extracted with hexane and analyzed by GLC through a 50 m x 0.32 mm CP-Wax58-CB fused-silica column (Chrompack), with methylheptadecanoic acid as an internal standard. The fatty acid content of

each fraction was quantified and used to calculate the relative amount of each lipid class. In order to determine the total lipid content, 3 ml aliquots from yeast cultures were harvested by centrifugation and the resulting pellets were washed with distilled water and lyophilized. The weight of the dried cells was determined and the fatty acid content was quantified by GLC-analyses after conversion to methylesters as described above. The lipid content was then calculated as nmol fatty acid (FA) per mg dry weight yeast.

Enzyme assays. Aliquots of crude microsomal fractions (corresponding to 10 nmol of microsomal PC) from developing plant seeds or yeast cells were lyophilized over night. ^{14}C-Labeled substrate lipids dissolved in benzene were then added to the dried microsomes. The benzene was evaporated under a stream of N_2, leaving the lipids in direct contact with the membranes, and 0.1 ml of 50 mM potassium phosphate (pH 7.2) was added. The suspension was thoroughly mixed and incubated at 30°C for the time period indicated, up to 90 min. Lipids were extracted from the reaction mixture using chloroform and separated by thin layer chromatography in hexane/diethylether/acetic acid (35:70:1.5) using silica gel 60 plates (Merck). The radioactive lipids were visualized and quantified on the plates by electronic autoradiography (Instant Imager, Packard, US).

Yeast cultivation. Yeast cells were grown at 28°C on a rotatory shaker in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose), synthetic medium (18) containing 2% (v/v) glycerol and 2% (v/v) ethanol, or minimal medium (19) containing 16 g/l of glycerol.

The instant invention is further characterized by the following examples which are not limiting:

Acyl-CoA-independent synthesis of TAG by oil seed microsomes. A large number of unusual fatty acids can be found in oil seeds (20). Many of these

fatty acids, such as ricinoleic (21) and vernolic acids (22), are synthesized using phosphatidylcholin (PC) with oleoyl or linoleoyl groups esterified to the *sn*-2 position, respectively, as the immediate precursor. However, even though PC can be a substrate for unusual fatty acid synthesis and is the major membrane lipids in seeds, unusual fatty acids are rarely found in the membranes. Instead, they are mainly incorporated into the TAG. A mechanism for efficient and selective transfer of these unusual acyl groups from PC into TAG must therefore exist in oil seeds that accumulate such unusual fatty acids. This transfer reaction was biochemically characterized in seeds from castor bean (*Ricinus communis)* and *Crepis palaestina,* plants which accumulate high levels of ricinoleic and vernolic acid, respectively, and sunflower (*Helianthus annuus)*, a plant which has only common fatty acids in its seed oil. Crude microsomal fractions from developing seeds were incubated with PC having ^{14}C-labeled oleoyl, ricinoleoyl or vernoloyl groups at the *sn*-2 position. After the incubation, lipids were extracted and analyzed by thin layer chromatography. We found that the amount of radioactivity that was incorporated into the neutral lipid fraction increased linearly over a period of 4 hours (data not shown). The distribution of [^{14}C]acyl groups within the neutral lipid fraction was analyzed after 80 min (Fig. 1). Interestingly the amount and distribution of radioactivity between diffferent neutral lipids were strongly dependent both on the plant species and on the type of [^{14}C]acyl chain. Thus, sunflower microsomes incorporated most of the label into DAG, regardless of the type of [^{14}C]acyl group. In contrast, *R. communis* microsomes preferentially incorporated [^{14}C]ricinoleoyl and [^{14}C]vernoloyl groups into TAG, while [^{14}C]oleyl groups mostly were found in DAG. *C. palaestina* microsomes, finally, incorporated only [^{14}C]vernolyol groups into TAG, with [^{14}C]ricinoleyl groups being found mostly as free fatty acids, and [^{14}C]oleyl groups in DAG. This shows that the high *in vivo* levels of ricinoleic acid and vernolic acid in the TAG pool of *R. communis* and *C. palaestina*, respectively, can be explained by an efficient and selective transfer of the corresponding acyl groups from PC to TAG in these organisms.

The in-vitro synthesis of triacylglycerols in microsomal preparations of developing castor bean is summarized in table 1.

PDAT: a novel enzyme that catalyzes acyl-CoA independent synthesis of TAG. It was investigated if DAG could serve both as an acyl donor as well as an acyl acceptor in the reactions catalyzed by the oil seed microsomes. Thererfore, unlabeled divernoloyl-DAG was incubated with either *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-DAG or *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC in the presence of *R. communis* microsomes. The synthesis of TAG molecules containing both [^{14}C]ricinoleoyl and vernoloyl groups was 5 fold higher when [^{14}C]ricinoleoyl-PC served as acyl donor as compared to [^{14}C]ricinoleoyl-DAG (fig.1B). These data strongly suggests that PC is the immediate acyl donor and DAG the acyl acceptor in the acyl-CoA-independent formation of TAG by oil seed microsomes. Therefore, this reaction is catalyzed by a new enzyme which we call phospholipid : diacylglycerol acyltransferase (PDAT).

PDAT activity in yeast microsomes. Wild type yeast cells were cultivated under conditions where TAG synthesis is induced. Microsomal membranes were prepared from these cells and incubated with *sn*-2-[^{14}C]-ricinoleoyl-PC and DAG and the ^{14}C-labeled products formed were analyzed. The PC-derived [^{14}C]ricinoleoyl groups within the neutral lipid fraction mainly were found in free fatty acids or TAG, and also that the amount of TAG synthesized was dependent on the amount of DAG that was added to the reaction (Fig.2). The *in vitro* synthesis of TAG containing both ricinoleoyl and vernoloyl groups, a TAG species not present *in vivo*, from exogenous added *sn*-2-[^{14}C]ricinoleoyl-PC and unlabelled vernoloyl-DAG (Fig. 2, lane 3) clearly demonstrates the existence of an acyl-CoA-independent synthesis of TAG involving PC and DAG as substrates in yeast microsomal membranes. Consequently, TAG synthesis in yeast can be catalyzed by an enzyme similar to the PDAT found in plants.

The PDAT encoding gene in yeast.

A gene in the yeast genome (YNR008w) is known, but nothing is known about the function of YNR008w, except that the gene is not essential for growth under normal circumstances. Microsomal membranes were prepared from the yeast strain FVKT004-04C(AL) (8) in which this gene with unknown function had been disrupted. PDAT activity in the microsomes were assayed using PC with radiolabelled fatty acids at the sn-2 position. The activity was found to be completely absent in the disruption strain (Fig. 2 lane 4). Significantly, the activity could be partially restored by the presence of YNR008w on the single copy plasmid pUS1 (Fig. 2 lane 5). Moreover, acyl groups of phosphatidylethanolamine (PE) were efficiently incorporated into TAG by microsomes from the wild type strain whereas no incorporation occured from this substrate in the mutant strain. This shows that YNR008w encodes a yeast PDAT which catalyzes the transfer of an acyl group from the *sn*-2 position of phospholipids to DAG, thus forming TAG. It should be noted that no cholesterol esters were formed from radioactive PC even in incubations with added ergosterols, nor were the amount of radioactive free fatty acids formed from PC affected by disruption of the YNR008w gene. This demonstrates that yeast PDAT do not have cholesterol ester synthesising or phospholipase activities.

Increased TAG content in yeast cells that overexpress PDAT. The effect of overexpressing the PDAT-encoding gene was studied by transforming a wild type yeast strain with the pUS4 plasmid in which the gene is expressed from the galactose-induced *GAL1:TPK2* promoter. Cells containing the empty expression vector were used as a control. The cells were grown in synthetic glycerol-ethanol medium, and expression of the gene was induced after either 2 hours (early log phase) or 25 hours (stationary phase) by the addition of galactose. The cells were then incubated for another 21 hours, after which they were harvested and assays were performed. We found that overexpression of PDAT had no significant effect on the growth rate as determined by the optical density. However, the total lipid content, measured as total µmol fatty acids per mg yeast dry weight, was 47% (log phase) or 29% (stationary phase) higher in the PDAT overexpressing strain than in the control. Furthermore, the polar lipid and sterolester content was unaffected by overexpression of PDAT. Instead, the elevated lipid content in these cells is entirely due to an increased TAG content (Fig. 3A,B). Thus, the amount of TAG was increased by 2-fold in PDAT overexpressing early log phase cells and by 40% in stationary phase cells. It is interesting to note that a significant increase in the TAG content was achieved by overexpressing PDAT even under conditions (*i.e.* in stationary phase) where DAGAT is induced and thus contributes significantly to TAG synthesis. *In vitro* PDAT activity assayed in microsomes from the PDAT overexpressing strain was 7-fold higher than in the control strain, a finding which is consistent with the increased levels of TAG that we observed *in vivo* (Fig. 3C). These results clearly demonstrate the potential use of the PDAT gene in increasing the oil content in transgenic organisms.

Substrate specificity of yeast PDAT. The substrate specificity of yeast PDAT was analyzed using microsomes prepared from the PDAT overexpressing strain (see Fig. 4). The rate of TAG synthesis, under conditions given in figure 4 with di-oleoyl-PC as the acyl-donor, was 0.15 nmol per min

and mg protein. With both oleoyl groups of PC labeled it was possible, under the given assay conditions, to detect the transfer of 11 pmol/min of [^{14}C]oleoyl chain into TAG and the formation of 15 pmol/min of lyso-PC. In microsomes from the PDAT-deficient strain, no TAG at all and only trace amounts of lyso-PC was detected, strongly suggesting that yeast PDAT catalyses the formation of equimolar amounts of TAG and lyso-PC when supplied with PC and DAG as substrates. The fact that somewhat more lyso-PC than TAG is formed can be explained by the presence of a phospholipase in yeast microsomes, which produces lyso-PC and unesterified fatty acids from PC (data not shown).

The specificity of yeast PDAT for different acyl group positions was investigated by incubating the microsomes with di-oleoyl-PC carrying a [^{14}C]acyl group either at the *sn*-1 position (Fig. 4A bar 2) or the *sn*-2 position (Fig. 4A bar 3). We found that the major ^{14}C-labeled product formed in the former case was lyso-PC, and in the latter case TAG. We conclude that yeast PDAT has a specificity for the transfer of acyl groups from the *sn*-2 position of the phospholipid to DAG, thus forming *sn*-1-lyso-PC and TAG. Under the given assay conditions, trace amounts of ^{14}C-labelled DAG is formed from the *sn*-1 labeled PC by the reversible action of a CDP-choline : choline phosphotransferase (data not shown). This labeled DAG can then be further converted into TAG by the PDAT activity. It is therefore not possible to distinguish whether the minor amounts of labeled TAG that is formed in the presence of di-oleoyl-PC carrying a [^{14}C]acyl group in the *sn*-1 position, is synthesized directly from the *sn*-1-labeled PC by a PDAT that also can act on the *sn*-1 postion, or if it is first converted to *sn*-1-labeled DAG and then acylated by a PDAT with strict selectivity for the transfer of acyl groups at the *sn*-2 position of PC. Taken together, this shows that the PDAT encoded by YNR008w catalyses an acyl transfer from the *sn*-2 position of PC to DAG, thus causing the formation of TAG and lyso-PC.

The substrate specificity of yeast PDAT was further analyzed with respect to the headgroup of the acyl donor, the acyl group transferred and the acyl chains of the acceptor DAG molecule. The two major membrane lipids of *S. cerevisiae* are PC and PE, and as shown in Fig. 4B (bars 1 and 2), dioleoyl-PE is nearly 4-fold more efficient than dioleoyl-PC as acyl donor in the PDAT-catalyzed reaction. Moreover, the rate of acyl transfer is strongly dependent on the type of acyl group that is transferred. Thus, a ricinoleoyl group at the *sn*-2 position of PC is 2.5 times more efficiently transferred into TAG than an oleoyl group in the same position (Fig. 4B bars 1 and 3). In contrast, yeast PDAT has no preference for the transfer of vernoloyl groups over oleoyl groups (Fig. 4B bars 1 and 4). The acyl chain of the acceptor DAG molecule also affects the efficiency of the reaction. Thus, DAG with a ricinoleoyl or a vernoloyl group is a more efficient acyl acceptor than dioleoyl-DAG (Fig. 4B bars 1, 5 and 6). Taken together, these results clearly show that the efficiency of the PDAT-catalyzed acyl transfer is strongly dependent on the properties of the substrate lipids.

PDAT genes. Nucleotide and amino acid sequences of several PDAT genes are given as SEQ ID No. 1 through 15. Futher provisional and/or partial sequences are given as SEQ ID NO 1a through 5a and 1b through 11b, respectively. One of the Arabidopsis genomic sequences (SEQ ID NO. 4) identfied an Arabidopsis EST cDNA clone; T04806. This cDNA clone was fully characterised and the nucleotide sequence is given as SEQ ID NO. 5. Based on the sequence homology of the T04806 cDNA and the *Arabidopsis thaliana* genomic DNA sequence (SEQ ID NO 4) it is apparent that an additional A is presesnt at position 417 in the cDNA clone (data not shown). Excluding this nucleotide would give the amino acid sequence depicted in SEQ ID NO. 12.

Increased TAG content in seeds of Arabidopsis thaliana that express the yeast PDAT. For the expression of the yeast pdat gene in *Arabidopsis thaliana* an EcoRI fragment from the pBluescript-pdat was cloned together with napin promotor (26) into the vector pGPTV-KAN (27). A plasmid (pGNapPDAT)

having the yeast PDAT gene in the correct orientation was identified and transformed into *Agrobacterium tumefaciens*. These bacteria were used to transform *Arabidopsis thaliana* columbia (C-24) plants using the root transformation method (28). Plants transformed with an empty vector were used as controls.

First generation seeds (T1) were harvested and germinated on kanamycin containing medium. Second generation seeds (T2) were pooled from individual plants and their fatty acid contents analysed by quantification of their methyl esthers by gas liquid chromatography after methylation of the seeds with 2% sulphuric acid in methanol at 85 °C for 1,5 hours. Quantification was done with heptadecanoic acid methyl esters as internal standard.

From the transformation with pGNapPDAT one T1 plant (26-14) gave raise to seven T2 plants of which 3 plants yielded seeds with statistically (in a mean difference two-sided test) higher oil content than seeds from T2 plants generated from T1 plant 32-4 transformed with an empty vector (table 2).

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Description of Figures

FIG. 1.

Metabolism of ^{14}C-labeled PC into the neutral lipid fraction by plant microsomes. (A) Microsomes from developing seeds of sunflower, *R. communis* and *C. palaestina* were incubated for 80 min at 30°C with PC (8 nmol) having oleic acid in its *sn*-1 position, and either ^{14}C-labeled oleic, ricinoleic or vernolic acid in its *sn*-2 position. Radioactivity incorporated in TAG (open bars), DAG (solid bars), and unsterified fatty acids (hatched bars) was quantified using thin layer chromatography followed by electronic autoradiography, and is shown as percentage of added labeled substrate. (B) Synthesis *in vitro* of TAG carrying two vernoloyl and one [^{14}C]ricinoleoyl group by microsomes from *R. communis*. The substrates added were unlabeled divernoloyl-DAG (5 nmol), together with either *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-DAG (0.4 nmol, 7700 dpm/nmol) or *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC (0.4 nmol, 7700 dpm/nmol). The microsomes were incubated with the substrates for 30 min at 30°C, after which samples were removed for lipid analysis as described in the section „general methods". The data shown are the average of two experiments.

FIG. 2.

PDAT activity in yeast microsomes, as visualized by autoradiogram of neutral lipid products separated on TLC. Microsomal membranes (10 nmol of PC) from the wild type yeast strain FY1679 (lanes 1-3), a congenic yeast strain (FVKT004-04C(AL)) that is disrupted for YNR008w (lane 4) or the same disruption strain transformed with the plasmid pUS1, containing the YNR008w gene behind its native promotor (lane 5), were assayed for PDAT activity. As substrates, we used 2 nmol *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC together with either 5 nmol of dioleoyl-DAG (lanes 2, 4 and 5) or *rac*-oleoyl-vernoleoyl-DAG (lane 3). The enzymatic assay and lipid analysis was performed as described in Materials and Methods. The cells were precultured for 20 h in liquid YPD

medium, harvested and re-suspended in an equal volume of minimal medium (19) containing 16 g/l glycerol. The cells were then grown for an additional 24 h prior to being harvested. Selection for the plasmid was maintained by growing the transformed cells in synthetic medium lacking uracil (18). Abbreviations: 1-OH-TAG, monoricinoleoyl-TAG; 1-OH-1-ep-TAG, monoricinoleoyl-monovernoloyl-TAG; OH-FA, unesterified ricinoleic acid.

Fig. 3.

Lipid content (A,B) and PDAT activity (C) in PDAT overexpressing yeast cells. The PDAT gene in the plasmid pUS4 was overexpressed from the galactose-induced *GAL1-TPK2* promotor in the wild type strain W303-1A (7). Its expression was induced after (A) 2 hours or (B) 25 hours of growth by the addition of 2% final concentration (w/v) of galactose. The cells were then incubated for another 22 hours before being harvested. The amount of lipids of the harvested cells was determined by GLC-analysis of its fatty acid contents and is presented as µmol fatty acids per mg dry weight in either TAG (open bar), polar lipids (hatched bar), sterol esters (solid bar) and other lipids (striped bar). The data shown are the mean values of results with three independent yeast cultures. (C) *In vitro* synthesis of TAG by microsomes prepared from yeast cells containing either the empty vector (vector) or the PDAT plasmid (+ PDAT). The cells were grown as in Fig. 3A. The substrate lipids dioleoyl-DAG (2.5 nmol) and *sn*-1-oleoyl-*sn*-2-[^{14}C]-oleoyl-PC (2 nmol) were added to aliquots of microsomes (10 nmol PC), which were then incubated for 10 min at 28 °C. The amount of label incorporated into TAG was quantified by electronic autoradiography. The results shown are the mean values of two experiments.

FIG. 4.

Substrate specificity of yeast PDAT. The PDAT activity was assayed by incubating aliquots of lyophilized microsomes (10 nmol PC) with substrate lipids at 30°C for 10 min (panel A) or 90 min (panel B). Unlabeled DAG (2.5 nmol) was used as substrates together with different labeled phospholipids, as shown

in the figure. (A) *Sn*-position specificity of yeast PDAT regarding the acyl donor substrate. Dioleoyl-DAG together with either *sn*-1-[^{14}C]oleoyl-*sn*-2-[^{14}C]oleoyl-PC (di-[^{14}C]-PC), *sn*-1-[^{14}C]oleoyl-*sn*-2-oleoyl-PC (*sn*1-[^{14}C]-PC) or *sn*-1-oleoyl-*sn*-2-[^{14}C]oleoyl-PC (*sn*2-[^{14}C]-PC). (B) Specificity of yeast PDAT regarding phospholipid headgroup and of the acyl composition of the phospholipid as well as of the diacylglycerol. Dioleoyl-DAG together with either *sn*-1-oleoyl-*sn*-2-[^{14}C]oleoyl-PC (oleoyl-PC), *sn*-1-oleoyl-*sn*-2-[^{14}C]oleoyl-PE (oleoyl-PE), *sn*-1-oleoyl-*sn*-2-[^{14}C]ricinoleoyl-PC (ricinoleoyl-PC) or *sn*-1-oleoyl-*sn*-2-[^{14}C]vernoloyl-PC (vernoloyl-PC). In the experiments presented in the 2 bars to the far right, monoricinoleoyl-DAG (ricinoleoyl-DAG or mono-vernoloyl-DAG (vernoloyl-DAG) were used together with *sn*-1-oleoyl-*sn*-2-[^{14}C]-oleoyl-PC. The label that was incorporated into TAG (solid bars) and lyso-PC (LPC, open bars) was quantified by electronic autoradiography. The results shown are the mean values of two experiments. The microsomes used were from W303-1A cells overexpressing the PDAT gene from the *GAL1-TPK2* promotor, as described in Fig. 3. The expression was induced at early stationary phase and the cells were harvested after an additional 24 h.

TAB.1:

<u>In vitro synthesis of triacylglycerols in microsomal preparations of developing castor bean.</u> Aliqouts of microsomes (20 nmol PC) were lyophilised and substrate lipids were added in benzene solution: (A) 0.4 nmol [^{14}C]-DAG (7760 dpm/nmol) and where indicated 1.6 nmol unlabelled DAG; (B) 0.4 nmol [^{14}C]-DAG (7760 dpm/nmol) and 5 nmol unlabelled di-ricinoleoyl-PC and (C) 0.25 nmol [^{14}C]-PC (4000 dpm/nmol) and 5 nmol unlabelled DAG. The benzene was evaporated by N_2 and 0.1 ml of 50 mM potassium phosphate was added, thoroughly mixed and incubated at 30 °C for (A) 20 min.; (B) and (C) 30 min.. Assays were terminated by extraction of the lipids in chloroform. The lipids were then separated by thin layer chromatography on silica gel 60 plates

(Merck; Darmstadt, Germany) in hexan/diethylether/acetic 35:70:1.5. The radioactive lipids were visualised and the radioactivity quantified on the plate by electronic autoradiography (Instant Imager, Packard, US). Results are presented as mean values of two experiments.

Radioactivity in different triacylglycerols (TAG) species formed. Abbreviations used: 1-OH-, mono-ricinoleoyl-; 2-OH, di-ricinoleoyl-; 3-OH-, triricinoleoyl; 1-OH-1-ver-, mono-ricinoleoly-monovernoleoyl-; 1-OH-2-ver-, mono-ricinoleoyl-divernoleoyl-. Radiolabelled DAG and PC were prepared enzymatically. The radiolabelled ricinoleoyl group is attached at the sn-2-position of the lipid and unlabelled oleoyl group at the sn-1-position. Unlabelled DAG with vernoleoyl- or ricinoleoyl chains were prepared by the action of TAG lipase (6) on oil of Euphorbia lagascae or Castor bean, respectively. Synthetic di-ricinoleoyl-PC was kindly provided from Metapontum Agribios (Italy).

TAB.2:

Total fatty acids per mg of T2 seeds pooled from individual *Arabidopsis thaliana* plants transformed with yeast PDAT gene under the control of napin promotor (26-14) or transformed with empty vector (32-4).

* = stastistical difference between control plants and PDAT transformed plants in a mean difference two-sided test at $\alpha = 5$.

Description of the SEQ ID:

SEQ ID NO. 1: Genomic DNA sequence and suggested amino acid sequence of the *Saccharomyces cerevisiae* PDAT gene, YNR008w, with GenBank accession number Z71623 and Y13139, and with nucleotide ID number 1302481.

SEQ ID NO. 2: The amino acid sequence of the suggested open reading frame YNR008w from *Saccharomyces cerevisiae.*

SEQ ID NO. 3: Genomic DNA sequence of the *Schizosaccharomyces pombe* gene SPBC776.14.

SEQ ID NO. 4: Genomic DNA sequence of part of the *Arabidopsis thaliana* locus with GenBank accession number AB006704.

SEQ ID NO. 5: Nucleotide sequence of the *Arabidopsis thaliana* cDNA clone with GenBank accession number T04806, and nucleotide ID number 315966.

SEQ ID NO. 6: Predicted amino acid sequence of the *Arabidopsis thaliana* cDNA clone with GenBank accession number T04806.

SEQ ID NO. 7: Nucleotide and amino acid sequence of the *Zea mays* EST clone with GenBank accession number AI491339, and nucleotide ID number g4388167.

SEQ ID NO. 8: Predicted amino acid sequence of the *Zea mays* EST clone with GenBank accession number AI491339, and nucleotide ID number g4388167.

SEQ ID NO. 9: DNA sequence of part of the *Neurospora crassa* EST clone W07G1, with GenBank accession number AI398644, and nucleotide ID number g4241729.

SEQ ID NO. 10: Genomic DNA sequence of part of the *Arabidopsis thaliana* locus with GenBank accession number AC004557.

SEQ ID NO. 11: Genomic DNA sequence of part of the *Arabidopsis thaliana* locus with GenBank accession number AC003027.

SEQ ID NO. 12: DNA sequencce of part of the *Lycopersicon esculentum* cDNA clone with GenBank accession number AI486635.

SEQ ID NO. 13: Amino acid sequence of the *Schizosaccharomyces pombe* putative open reading frame CAA22887 of the *Schizosaccharomyces pombe* gene SPBC776.14.

SEQ ID NO. 14: Amino acid sequence of the *Arabidopsis thaliana* putative open reading frame AAC80628 derived from the *Arabidopsis thaliana* locus with GenBank accession number AC004557.

SEQ ID NO 15: Amino acid sequence of the *Arabidopsis thaliana* putative open reading frame AAD10668 derived from the *Arabidopsis thaliana* locus with GenBank accession number AC003027.

Further provisional and/or partial sequences are defined through the following SEQ IDs:

SEQ ID NO. 1a: The amino acid sequence of the yeast ORF YNR008w from *Saccharomyces cerevisiae*.

SEQ ID NO. 2a: Amino acid sequence of the region of the *Arabidopsis thaliana* genomic sequence (AC004557).

SEQ ID NO. 3a: Amino acid sequence of the region of the *Arabidopsis thaliana* genomic sequence (AB006704).

SEQ ID NO. 4a: The corresponding genomic DNA sequence and amino acid sequence of the yeast ORF YNROO8w from *Saccharomyces cerevisiae.*

SEQ ID NO. 5a: The amino acid sequence of the yeast ORF YNROO8w from *Saccharomyces cerevisiae* derived form the corresponding genomic DNA sequence.

SEQ ID NO. 1b: Genomic DNA sequence of the *Saccharomyces cerevisiae* PDAT gene, YNR008w, genebank nucleotide ID number 1302481, and the suggested YNR008w amino acid sequence.

SEQ ID NO. 2b: The suggested amino acid sequence of the yeast gene YNR008w from *Saccharomyces cerevisiae.*

SEQ ID NO. 3b: Genomic DNA sequence of the *Schizosaccharomyces pombe* gene SPBC776.14.

SEQ ID NO. 4b: Genomic DNA sequence of part of the *Arabidopsis thaliana* locus with genebank accession number AB006704.

SEQ ID NO. 5b: Nucleotide sequence and the corresponding amino acid sequence of the *Arabidopsis thaliana* EST-clone with genebank accession number T04806, and ID number 315966.

SEQ ID NO. 6b: Nucleotide and amino acid sequence of the *Zea mays* cDNA clone with genebank ID number g4388167.

SEQ ID NO. 7b: Amino acid sequence of the *Zea mays* cDNA clone with genebank ID number g4388167.

SEQ ID NO. 8b: DNA sequence of part of the *Neurospora crassa* cDNA clone WO7G1, ID number g4241729.

SEQ ID NO. 9b: Genomic DNA sequence of part of the *Arabidopsis thaliana* locus with genebank accession number AC004557.

SEQ ID NO. 10b: Genomic DNA sequence of part of the *Arabidopsis thaliana* locus with genebank accession number AC003027.

SEQ ID NO. 11b: DNA sequence of part of the *Lycopersicon esculentum* cDNA clone with genebank accession number AI486635.